

A NOVEL POTASSIUM CHANNEL MODULATOR PEPTIDE

FIELD OF INVENTION

5 This invention pertains to the field of potassium channel modulators that have utility as therapeutic molecules for diseases such as cardiac arrhythmias, multiple sclerosis, a rheumatoid arthritis and other syndromes involving defective potassium channels.

PRIOR ART

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Potassium channels are membrane proteins, which determine the resting membrane potential and the duration of action potentials, involved in complex biological processes such as regulation of heart rate, muscle contraction, neuronal excitability and immune function (Kurokawa, J. *et al.*, *Journal of Molecular and Cellular Cardiology* (2001), 33, 873-882; Dodson, P.D. and Forsythe, I.D., *Trends in Neurosciences* (2004), 27, 210-217; 15 Koo, G.C. *et al.*, *Cellular Immunology* (1999), 197, 99-107). They act as targets for neurotransmitters, drugs, toxins etc. and are attractive tool for rational drug design. For example, blockers of certain K⁺ channels show class III antiarrhythmias action, a property being utilized for preventing or suppressing re-entrant arrhythmias (Tamargo, J. 20 *et al.*, *Cardiovascular Research* (2004), 62, 9-33). Margatoxin, a scorpion derived toxin, is reported to have a property to treat autoimmune disorders such as multiple sclerosis and rheumatoid arthritis (Shak, K *et al.*, *Cellular Immunology* (2003), 221, 100-106). Conotoxins, peptides from cone snails, are among the most potent ligands for receptors and channels in the central nervous system. A kappa conotoxin, κ -Pviiia, from *Conus* 25 *purpurascens*, has been advanced as a possible candidate for treatment of Alzheimer's disease, Lambert-Eaton syndrome and myasthenia gravis (Olivera, B.M. *et al.*, *Expert Opinion on Therapeutic Patents* (2001), 11, 603- 623). Potassium channels are membrane proteins, which determine the resting membrane potential and the duration of action potentials, involved in complex biological processes such as regulation of heart rate, 30 muscle contraction, neuronal excitability and immune function (Kurokawa, J. *et al.*, *Journal of Molecular and Cellular Cardiology* (2001), 33, 873-882; Dodson, P.D. and Forsythe, I.D., *Trends in Neurosciences* (2004), 27, 210-217; Koo, G.C. *et al.*, *Cellular*

Immunology (1999), 197, 99-107). They act as targets for neurotransmitters, drugs, toxins etc. and are attractive tool for rational drug design. For example, blockers of certain K⁺ channels show class III antiarrhythmias action, a property being utilized for preventing or suppressing re-entrant arrhythmias (Tamargo, J. *et al.*, *Cardiovascular Research* (2004), 62, 9-33). Margatoxin, a scorpion derived toxin, is reported to have a property to treat autoimmune disorders such as multiple sclerosis and rheumatoid arthritis (Shak, K *et al.*, *Cellular Immunology* (2003), 221, 100-106). Conotoxins, peptides from cone snails, are among the most potent ligands for receptors and channels in the central nervous system. A kappa conotoxin, κ -Pviiia, from *Conus purpurascens*, has been advanced as a possible candidate for treatment of Alzheimer's disease, Lambert-Eaton syndrome and myasthenia gravis (Olivera, B.M. *et al.*, *Expert Opinion on Therapeutic Patents* (2001), 11, 603-623).

The molecular diversity of K⁺ channels is larger than any other group of ion channels, with more than 80 different genes and many splice variants. The diversity is strikingly observed in the central nervous system, with numerous subtypes of neurons expressing a unique set of potassium channels. The voltage gated potassium channels are responsible for the repolarization of the action potential in neurons. Earlier electrophysiological studies on dorsal root ganglion neurons have indicated the expression of at least six voltage gated K⁺ currents, three transient and three non- inactivating currents.

SUMMARY OF INVENTION

The instant invention claims novel potassium channel modulating activity for a 13-residue peptide obtained from *Conus monile*. The target sequence has been prepared by conventional solid phase peptide synthesis procedure and found identical to the natural peptide. Analogues of this peptide sequence can be readily prepared by synthetic methods.

Electrophysiological studies suggest that Mo1659, a 13-residue peptide, specifically acts on the non-inactivating K⁺ currents. Studies with cloned K⁺ channels and investigations

of synthetic analogues are necessary to identify the target channel subtype and to establish the molecular mechanism of channel blocking activity.

DETAILED DESCRIPTION OF INVENTION

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The instant invention discloses a substantially pure peptide having the amino acid sequence FHGGSWYRFPWGY (SEQ ID NO: 1).

The peptide is used a potassium channel modulator.

A process of preparing substantially pure peptide comprising of:

- 10 (i) isolation of the peptide, and
 (ii) purifying the peptide by chromatographic methods.

The peptide in step (i) is isolated from venoms of *Conus monile*.

The purification step (ii) is carried out by HPLC (High Performance Liquid Chromatography).

- 15 The peptide is used for treatment neurophysiological and neurological disorders.

The peptide is used for treatment neurophysiological and neurological disorders in schizophrenia, epilepsy, bipolar disorder or in syndromes that affect the nervous system.

A pharmaceutical composition comprising a peptide having the amino acid sequence FHGGSWYRFPWGY (SEQ ID NO: 1) with or without pharmaceutically acceptable
20 carriers.

The invention will now be discussed in the following examples, not to be considered as limiting.

EXAMPLES

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EXAMPLE 1 (Isolation and purification of peptide)

The specimen, *Conus monile* was collected from the southeast coast of India. The venom ducts after dissection were preserved in ethanol and the venom that oozes out was
30 subjected to High Performance Liquid Chromatography (HPLC) purification after concentration on a rotavapor. Crude venom extract was applied onto a Jupiter 4 μ , Proteo 90Å, C₁₈ column (10 mm x 250 mm) and eluted with a linear gradient of acetonitrile

containing 0.1% TFA. The flow rate was maintained at 1 ml min^{-1} and the absorbance was monitored at 226 nm. Fractionation into several peptide components was achieved. The peptide components were analyzed by MALDI mass spectra analysis of individual HPLC fractions. The intense component at the retention time of 23.4 minutes corresponding to a molecular mass of 1659 Da was chosen for mass spectrometric de novo sequencing. The peptide component showed a high resolution MALDI mass spectrum, which establishes $[M+H]^+ = 1659.1$ Da (monoisotopic mass). The inset shows the charge states observed in an electrospray mass spectrum, where the +2 and +3 states are detectable suggesting the presence of at least three protonatable groups in the molecule. Attempted reduction with DTT followed by alkylation with iodoacetamide left the molecular mass unchanged, establishing the *absence* of disulfide bonds. Acetylation with acetic anhydride and acetic acid yielded a product with a mass $[M+H]^+ = 1701.3$ Da ($\Delta m = +42\text{ Da}$) indicating the presence of a single primary amino group. UV and fluorescence spectra established the presence of both Trp and Tyr residues. Peptide sequencing was undertaken using MALDI MS/MS techniques selecting the 1659.1 Da as the precursor ion. Figure 3 shows the observed fragment ions along with assignments of the b and y ion series (13). The presence of an intense b2 ion at 285 Da permitted sequential tracing of the 8-residue segment –GGSWYRFP–(SEQ ID NO: 3). The immonium ions at 70, 110, 136 and 159 suggested the presence of the residues Pro, His, Tyr and Trp, respectively. The b2 ion at 285 Da could correspond to the dipeptide –FH– or –HF– at the amino terminus. The observation of mass peaks at 194.9 Da suggested the presence of the dipeptide ion –GH– or –HG–. This supports the assignment of the sequence –FHG– at the N-terminus. The paucity of intense fragments in the mass range 1200 – 1500 Da limited extension of the sequence at the C-terminus.

EXAMPLE 2 (Conformation of the peptide)

Confirmation of the determined sequence was achieved by two independent methods. First, a synthetic peptide corresponding to the determined sequence of Mo1659 was prepared and its MS/MS fragmentation pattern shown to be identical to that of the natural product (Figure 5). The identity of the synthetic and natural peptides was also established by HPLC analysis (Figure 5, inset). Second, conventional Edman sequencing using an

automated sequenator confirmed the sequence. A notable feature of Mo1659 is the presence of as many as seven aromatic amino acids (F-2, Y-2, W-2, H-1) in a short stretch of 13 residues. The positively charged peptide is notably deficient in the common aliphatic, hydrophobic amino acids like Ala, Val, Leu and Ile.

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EXAMPLE 2(a)

Reduction and alkylation: The purified peptide was dissolved in 30 μ L, 0.05 M NH_4HCO_3 buffer, pH 8.0. For the reduction, 200 mM stock dithiothreitol (DTT) was added to a final concentration of 8 mM and incubated at 37 $^\circ\text{C}$ for 2 h followed by addition of iodoacetamide stock solution, to get a final concentration of 40 mM. The resulting mixture was incubated at room temperature in the dark, for 1 h and subsequently analyzed by MALDI MS.

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Acetylation: The peptide solution was dried and resuspended in a 1:1 ratio of acetic acid and acetic anhydride. After 5 min of incubation at room temperature, the resultant solution was again dried and resuspended in 0.1% TFA and acetonitrile in the ratio 1:1 (v/v) and analyzed using MALDI MS. Trypsin digestion The purified sample was digested with TPCK treated trypsin (Sigma Co., USA) with 10 μ g of enzyme in 50 μ l of 50 mM NH_4HCO_3 , pH 8.0 for 3 h at 37 $^\circ\text{C}$. The digest was analyzed using MALDI and ESI mass spectrometers.

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Trypsin digestion : The purified sample was digested with TPCK treated trypsin (Sigma Co., USA) with 10 μ g of enzyme in 50 μ l of 50 mM NH_4CO_3 , pH 8.0 for 3 h at 37 $^\circ\text{C}$. The digest was analyzed using MALDI and ESI mass spectrometers.

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Digestion of Mo1659 with trypsin yielded two fragments with masses (MALDI) 1010 Da and 668 Da, corresponding to the N-terminus and C-terminus fragments, respectively. The 668 Da fragment is assigned to the C-terminus fragment -FPXX (SEQ ID NO: 4), which has previously been detected as a fragment ion in Figure 3 [Note that 17 Da (OH) is added to the N-terminus fragment and 1 Da (H) to the C-terminus fragment, upon hydrolysis]. The 668 Da fragment was subjected to high-energy collision induced dissociation (CID) in an ion trap mass spectrometer, using an electrospray ionization source. The ESI MS/MS fragmentation pattern observed is shown in Figure 4. The

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intense peak at 651 Da corresponds to a facile loss of NH₃ from the C-terminus suggestive of the presence of C-terminal amidation. *Conus* peptides are often posttranslationally modified, with amidation being commonly observed (14,15). The identification of the b₂ ion at 245 Da corresponds to the -FP- fragment, already
5 established by MALDI MS/MS, permitting the ready identification of the C-terminus tripeptide as -WGY-amide. The final determined sequence is FHGGSWYRFPWGY-NH₂ (SEQ ID NO: 1), corresponding to a calculated average mass of 1659.8 Da (Average mass observed in ESI MS = 1659.3 Da).

10 **Mass spectrometry** : Electrospray ionization (ESI) mass spectra were obtained on a Hewlett Packard, HP 1100 MSD series spectrometer equipped with a single quadrupole analyzer. The data were acquired over a range of 300 to 1500 m/z in positive ion mode and analyzed using HP LC/MSD Chemstation software. MALDI spectra were collected
15 using a Bruker Daltonics, Ultraflex TOF/TOF system, in the reflectron positive ion mode, equipped with a nitrogen laser of 337 nm. The samples were prepared by mixing equal volumes of peptide solution and saturated matrix (α -cyano-4-hydroxy cinnamic acid). A standard peptide mixture was used for external calibration. ESI MS/MS data were obtained on Esquire 3000 plus LC ion trap mass spectrometer (Bruker Daltonics, Germany). The nitrogen gas pressure and flow rate to the nebulizer were 10 bar and 5 l
20 min⁻¹, respectively with a drying gas temperature of 300 °C. The scan range was set at 50 to 1000 m/z. The sample was dissolved in 1:1(v/v) ratio of water and acetonitrile containing 0.1% HCOOH and was infused directly into the system delivered by a syringe pump (Cole-Parmer, Vernon Hills, Illinois, USA) at a flow rate of 120 μ l h⁻¹. Helium was used as the collision gas for CID experiments. The data were analyzed using Esquire
25 data analysis software, version 3.1.

Edman sequencing: The primary sequence of the peptide was determined by using a Shimadzu PPSQ-10 protein sequencer equipped with an LC-10A HPLC system.

30 **EXAMPLE 3 (Confirmation of Mo 1659 sequence)**

Confirmation of the determined sequence was achieved by two independent methods. First, a synthetic peptide corresponding to the determined sequence of Mo1659 was prepared and its MS/MS fragmentation pattern shown to be identical to that of the natural product (Figure 5). The identity of the synthetic and natural peptides was also established
5 by HPLC analysis (Figure 5, inset). Second, conventional Edman sequencing using an automated sequenator confirmed the sequence. A notable feature of Mo1659 is the presence of as many as seven aromatic amino acids (F-2, Y-2, W-2, H-1) in a short stretch of 13 residues. The positively charged peptide is notably deficient in the common aliphatic, hydrophobic amino acids like Ala, Val, Leu and Ile.

EXAMPLE 4 (Novelty of the conopeptide Mo 1650)

A search of the existing protein sequence databases reveals no matches for the determined sequence of Mo1659. The uniqueness of the peptide is established through
15 different database searches, using different algorithms like Blast, BlastP etc as described below :

Uniqueness of the sequence: FHGGSWYRFPWGY (SEQ ID NO: 1)

Uniqueness of the sequence FHGGSWYRFPWGY (SEQ ID NO: 1) was shown in the
20 following ways:

Prosite was used to search for the sequence:

F-H-G-G-S-W-Y-R-F-P-W-G-Y (SEQ ID NO: 1)

25 This sequence was searched for in PDB, Swissprot, TrEMBL, TrEMBL new. No hits were found.

Prosite was also used to search for the following variant:

[FY]-H-G-G-S-W-[YF]-[RK]-[FY]-P-W-G-[YF] (SEQ ID NO: 2)

30 No hits were found.

EXAMPLE 5 (Assay For Potassium Channel Activation Of Peptides)

DRG neuron preparation Dorsal root ganglion (DRG) neurons for electrophysiological studies were prepared as follows :

5th Postnatal day male Wistar rats were anesthetized with diethyl ether. The whole vertebral column was removed and transferred to a dish containing pre-oxygenated phosphate buffered saline. While holding the vertebral column a strip of bone from the dorsal root of the vertebral column was cut. The dorsal root ganglia together with dorsal and ventral roots were individually taken out with fine dissecting forceps and transferred into phosphate buffered saline containing 1.5 mg ml⁻¹ trypsin (from porcine pancreas, Sigma, USA). The DRGs were minced with dissecting spring scissors, and incubated at 37 °C for 30 min. After trypsin treatment, the cells were pelleted by centrifugation at 1000 rpm. for 5 min. The supernatant was removed, and washed with 1 ml of DMEM containing 10% FBS. Following resuspension in fresh DMEM containing 10% FBS, single cell suspension was obtained by trituration using a fire polished Pasteur pipette. To increase the cell density, a locally fabricated 8 mm diameter optically polished glass ring was placed on the bottom of a sterile 35 mm tissue culture dish. The suspended cells were plated into the well formed by the glass ring. The cells were incubated for 1 h at 37 °C. Isolated DRG neurons were used for the electrophysiology experiments.

Electrophysiology: Isolated K⁺ currents were recorded from DRG neurons using the patch-clamp technique in the whole-cell mode using an EPC-8 amplifier (Heka). Patch-clamp electrodes with resistance of 1-3 mega-ohm were made from borosilicate glass (Clark Electromedical Instruments, UK). The external bath solution contained: 130 mM choline chloride, 3 mM KCl, 2.5 mM CoCl₂, 0.6 mM MgCl₂, 10 mM Hepes, 1.2 mM NaHCO₃ and 10 mM glucose, pH 7.4 with Tris base; osmolarity, 325 mosmol adjusted with sucrose. The internal solution contained: 140 mM KCl, 1 mM CaCl₂, 2H₂O, 2 mM MgCl₂.6H₂O, 11 mM EGTA, 10 mM Hepes, pH 7.2 with Tris base; osmolarity 310 mosmol. The neurons were voltage-clamped at -80 mV in all the experiments. Capacity and leak subtraction was done using a P/4 subtraction protocol. Using Rs compensation

of 50% in all the experiments minimized voltage errors. Data acquisition and pulse protocols were controlled with the pClamp8 software, and Digidata 1320 analog/digital converter (Axon Instruments Inc.). The bath temperature was maintained at 20 °C. The toxin was dissolved in water. Bolus application of the toxin was employed to achieve a
5 final bath concentration of 200 nM. The effects of Mo1659 on the K⁺ currents reported here were recorded 15 min after peptide application.

Mo1659 shows K⁺ channel modulating activity in DRG neurons. Figure 6 shows the effect of Mo1659 on the mixed whole-cell outward K⁺ currents from a DRG neuron. A
10 marked reduction in the current amplitudes at all the potentials was observed with 200 nM of the Mo1659 in the external bath solution. The mixed K⁺ currents have a fast transient current and a sustained current component. The fast transient current component was dissected from the sustained current component using two different pre-pulse voltages followed by identical voltage protocols that are shown schematically in Figure 7.
15 Although the K⁺ current components cannot be fully isolated using the conditioning prepulse voltages alone (18), the results suggest that Mo1659 addition to the external bath solution predominantly affects the sustained K⁺ current component. It may be noted that the transient current component, that was obtained following subtraction of current traces, is not significantly affected by Mo1659. Similar results were obtained in 5
20 different experiments. Mo1659 thus appears to affect non-inactivating voltage dependent potassium channels. The reduction of total K⁺ currents in excitable cells by blocking potassium channels is a process, which is important in developing therapeutics for arrhythmias and heart failure. Enhancing the duration of action potentials using potassium channel blockers is a possible strategy for the development of new classes of
25 antiarrhythmic agents.

EXAMPLE 6 (Process Of Producing Monile Peptide Mo 1659)

Purification from the snail venom : The specimen, *Conus monile* was collected from
30 the southeast coast of India. The venom ducts after dissection were preserved in ethanol and the venom that oozes out was subjected to High Performance Liquid Chromatography (HPLC) purification after concentration on a rotavapor. Crude venom

extract was applied onto a Jupiter 4 μ , Proteo 90Å, C₁₈ column (10 mm x 250 mm) and eluted with a linear gradient of acetonitrile containing 0.1% TFA. The flow rate was maintained at 1ml min⁻¹ and the absorbance was monitored at 226 nm. The intense component at the retention time of 23.4 minutes is the desired Mo1659 peptide. The homogeneity of this fraction was demonstrated by re analysis using HPLC on an analytical column (Zorbax C18 RP, 300Å pore size, 5 μ particle size). Furthermore, identity of the peptide and the absence of contaminants were demonstrated by LC- ESI and MALDI mass spectrometry.

- 10 The peptide thus obtained is tested for electrophysiological activity and stored at +4 °C for further use.

EXAMPLE 7 (Chemical Synthesis Of Monile Peptide, Mo1659)

The peptide was synthesized by standard solid phase peptide synthetic methods using Fmoc chemistry. All amino acids are protected at the N-terminus with the Fmoc group (Nova Biochem). The side chains of Tyr and Ser were protected with the t-Bu group, Arg with Mtr group, His with trityl group. The coupling reactions proceeded using the OPfp esters of the protected amino acids on Fmoc-Rink amide AM resin (200-400 mesh, Nova Biochem). The synthesis was performed with 300 mg of resin with a bead capacity of 0.63 mMg⁻¹. The C-terminal amino acid (Tyr) was linked to the resin by the formation of an amide linkage with the amino functional group emanating from the solid support. Ser and His were coupled by using HBTU (N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium hexafluoro phosphate N-oxide). The Fmoc deprotections were performed with 20% piperidine in dimethylformamide. Peptide was cleaved from 100 mg of resin after synthesis, using 94% TFA (7.52 ml), containing 5% anisole (400 μ l) and 1% ethanedithiol (80 μ l) as cation scavengers. After 5-6 h, the resin was filtered off, the TFA was removed by evaporation in vacuo and the peptide was precipitated with ether. The precipitate was repeatedly washed with ether and purified by RP HPLC.

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It is also possible to produce this peptide by recombinant DNA technology taking advantage of the fact that this invention describes the peptide sequence and based on

which DNA sequence can be derived from the known triplet codes for each amino acid. The DNA sequence thus obtained can be synthesized/relevant gene stretch can be obtained from the snail DNA using methods such cDNA cloning, Polymerase chain reaction etc and cloned into expression vectors either in prokaryotic or eukaryotic systems. The clones thus obtained can be engineered to produce the peptide Mo 1659 by known methods and purified to homogeneity by known methods.